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# Antibody Formation in Mouse Bone Marrow

## I. EVIDENCE FOR THE DEVELOPMENT OF PLAQUE-FORMING CELLS *IN SITU*

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**Summary.** Mouse bone marrow as a source of plaque-forming cells (PFC) was studied by single and multiple intravenous injections of sheep erythrocytes (SRBC). In the primary response a great number of PFC appeared in the spleen while only a few showed up in the marrow. In the secondary response there is a clear PFC-response in both the spleen and the bone marrow. The spleen contains the majority of PFC until about 9 days after the second injection. In the course of the reaction, however, the number of PFC in the bone marrow rises to a level which surpasses the level in the spleen. IgM-PFC as well as IgG-PFC and IgA-PFC could be demonstrated in the marrow. In the lymph nodes and Peyer's patches the number of PFC did not increase above the normal background level at any time after the primary or secondary immunization. In order to test whether the bone marrow PFC-response is caused by a migration of PFC from the spleen or by development *in situ*, mice were splenectomized shortly before the second injection of SRBC. It could be shown that splenectomy does not prevent the bone marrow PFC-response. Because no activity in the other lymphoid organs was observed, it is concluded that the PFC activity of the marrow is caused by development *in situ*.

## INTRODUCTION

Antibody- and $\gamma$-globulin synthesis can take place in bone marrow. In the early part of the century it was stated that the bone marrow of immunized guinea-pigs (Lüdke, 1912) and rabbits (Lüdke, 1912; Reiter, 1913) is capable of antibody-production *in vitro*. In recent years antibody- and $\gamma$-globulin synthesis in the bone marrow were reported for guinea-pig (Askonas and White, 1956; Askonas, White and Wilkinson, 1965; Fleming, Wilkinson and White, 1967), rabbit (Thorbecke and Keuning, 1953, 1956; Askonas and Humphrey, 1958; Thorbecke, 1960; Thorbecke, Asofsky, Hochwald and Siskind, 1962), human (Asofsky and Thorbecke, 1961; van Furth, 1966; McMillan, Longmire, Yelenosky, Lang, Heath and Craddock, 1972) and monkey (Asofsky and Thorbecke, 1961) bone marrow *in vitro*.

Studies in rabbits (Kolouch, Good and Campbell, 1947) and in man (Good, 1955) provided evidence for a positive correlation between the plasma cell content of the bone marrow and the serum titre after intensive antigenic stimulation. Recently Hijmans, Schuit and Hulsing-Hesselink (1971) and Silverman, Yagi, Pressman, Ellison and

Tormey (1973) reported studies of human bone marrow cells analysed for the presence of immunoglobulins with the fluorescent antibody technique. The $\kappa/\lambda$ ratio and the percentage distribution of cells of the lymphoid series which were positive for the heavy chains of the major classes ($\alpha$, $\mu$ and $\gamma$) showed a striking similarity with the $\kappa/\lambda$ ratio in the serum and with the percentage distribution of the serum immunoglobulins, if corrected for pool size and metabolic rate. Hijmans and Schuit (1972) concluded that in humans bone marrow is a major source of immunoglobulins.

So far mouse bone marrow has not been demonstrated to contain a substantial number of plaque-forming cells (PFC) after immunization (Friedman, 1964; Eidinger and Pross, 1967; Chaperon, Selner and Claman, 1968; Mellbye, 1971; Cohen, 1972; Anderson and Dresser, 1972). However, some activity in the bone marrow was found with an increasing interval after immunization with sheep red cells (SRBC) (Eidinger and Pross, 1967; Chaperon *et al.*, 1968; Mellbye, 1971; Anderson and Dresser, 1972).

We have studied the primary and secondary response against SRBC by the haemolytic plaque assay in mouse bone marrow. The activity of the marrow was compared with the activity of the other lymphoid organs. We have shown that after two intravenous injections with SRBC, mouse bone marrow contains a considerable part of the total number of PFC. The presence of these large numbers of PFC in bone marrow may be due to a migration of PFC from the spleen. Such transport is suggested in the literature (Langevoort, Asofsky, Jacobsen, de Vries and Thorbecke, 1963, van Furth *et al.*, 1966; Chaperon *et al.*, 1968; Hijmans and Schuit, 1972). We tested this possibility by studying the secondary response of bone marrow in mice, splenectomized shortly before their second injection of SRBC.

## MATERIALS AND METHODS

### *Mice*

(C57Bl/Rij × CBA/Rij) $F_1$ female mice, 20 weeks old were used. They were purchased from the Medical Biological Laboratory (Rijswijk, The Netherlands).

### *Immunization*

For primary immunization, the mice received an intravenous injection of $4 \times 10^8$ SRBC. This injection was repeated in 5 weeks for the secondary immunization.

### *Splenectomy*

Spenectomy and sham-splenectomy were performed 1 day before the second injection of SRBC. The mice were anaesthetized with an intraperitoneal injection of 70 mg/kg body weight Nembutal (Abbott S. A., Saint-Rémy-sur-Avre, France). There was no post-operative mortality.

### *Preparation of cell suspensions*

At various times following immunization four or five animals were killed. Immediately after killing the mice with ether, the spleens, femurs, peripheral lymph nodes (inguinal, brachial and axillary), mesenteric lymph nodes and Peyer's patches were removed and brought into a balanced salt solution (BSS). This solution was prepared according to Mishell and Dutton (1967) and was always supplemented with 5 per cent newborn calf serum.

Bone marrow was obtained by flushing the femurs with BSS. Spleens, lymph nodes,

Peyer's patches and bone marrow were minced with scissors and squeezed through a nylon-gauze filter to give a single cell suspension.

*Assay for PFC*

The method for detection of PFC developed by Cunningham and Szenberg (1968) was adopted with some modifications as described by Zaalberg, van der Meul and Twisk (1968). One coverslip of 60 × 24 mm was used per microscope slide. The maximum concentration of cell suspension was $2 \times 10^7$ cells/ml and a volume of 0·4 ml was used for each test. The chambers were incubated at 37° for 2 hours. The number of IgM-PFC was calculated from a direct assay in which the slides were developed with guinea-pig complement (Flow Laboratories, Rockville, U.S.A.) only. The number of IgM-PFC+ IgG-PFC was calculated from an indirect assay in which the slides were developed with guinea-pig complement and rabbit-anti-mouse-IgG. The number of IgG-PFC was obtained by subtracting the number of PFC obtained in the direct assay from those obtained in the indirect assay. The number of IgA-PFC was calculated in a similar way employing rabbit-anti-mouse-IgA. From the results of the femoral bone marrow the number of PFC present in the marrow of the whole animal was estimated, using the data of Chervenick, Boggs, March, Cartwright and Wintrobe (1968), who showed that in mice one femur contains 5·9 per cent of the marrow.

*Hyperimmune serum*

For hyperimmune-anti-SRBC serum (C57Bl/Rij × CBA/Rij) $F_1$ female mice received four injections of $4 \times 10^8$ SRBC. The first injection was given intravenously, and subsequent ones were administered every other week intraperitoneally. The agglutination titre of the serum used was $1:2^{16}$.

*Statistics*

The standard deviation (S.D.) associated with $p$ plaques counted was calculated as the square root of $p + 0{\cdot}004 \times p^2$. This formula was drawn up by Jerne for the agar plaque assay. For calculation of the 95 per cent confidence limits the formula $p+$ two S.D. was used. The presence of IgG and IgA was accepted as significant when there was no overlap between the upper limit in the direct assay and the lower limit in the indirect assay.

## RESULTS

### PRIMARY RESPONSE

After a single i.v. injection of $4 \times 10^8$ SRBC the main PFC-activity could be observed in the spleen (Fig. 1). Only slight activity was detected in the bone marrow; during the early response some IgM-PFC appeared, but were not detected in significant quantities. However, after 27 days small numbers of IgM-, IgG- and IgA-PFC could be found in the marrow. In the peripheral lymph nodes, the mesenteric lymph node, and Peyer's patches the presence of PFC above the normal background level could not be demonstrated.

### SECONDARY RESPONSE

A second i.v. injection of $4 \times 10^8$ SRBC, given 5 weeks after the first one, resulted in

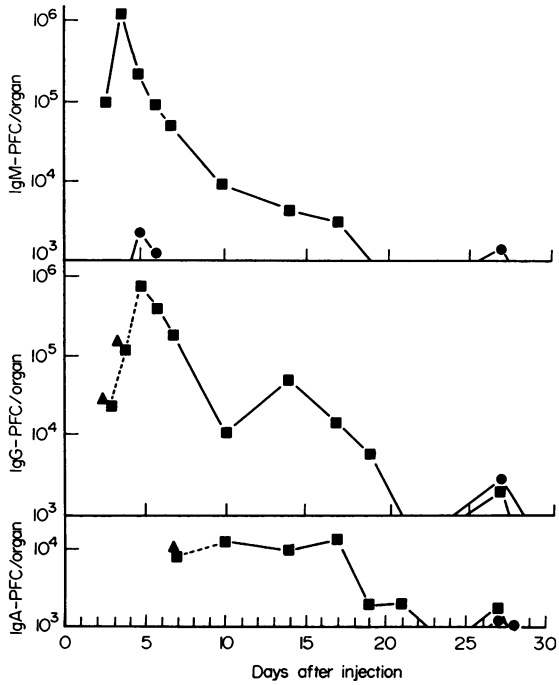


FIG. 1. Number of PFC in mouse spleen and bone marrow after an i.v. injection of $4 \times 10^8$ SRBC. (■) Spleen and (●) bone marrow. Where (▲) is added to an experimental point it means that the number of IgG-PFC or IgA-PFC above the level of IgM-PFC was not significant.

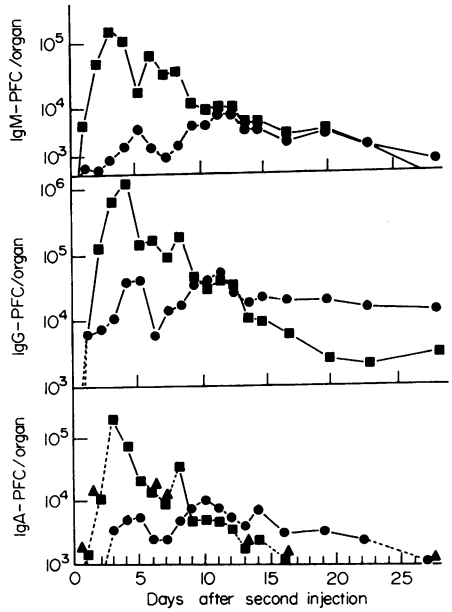


FIG. 2. Number of PFC in mouse spleen and bone marrow after two i.v. injections of $4 \times 10^8$ SRBC. The second injection was given 5 weeks after the first. (■) Spleen and (●) bone marrow. Where (▲) is added to an experimental point it means that the number of IgG-PFC or IgA-PFC above the level of IgM-PFC was not significant.

the rapid appearance of IgM-, IgG- and IgA-PFC in the spleen as well as in the bone marrow (Fig. 2). During the first 9–10 days the spleen contained the majority of the PFC; however, in the course of the reaction the bone marrow became more important as a source of PFC. After the 9th day the bone marrow contained as many IgM-, IgG- and IgA-PFC as the spleen. After the 13th day IgG-PFC in the bone marrow were even more numerous. The number of PFC in peripheral lymph nodes, mesenteric lymph node and Peyer's patches remained at the normal background level.

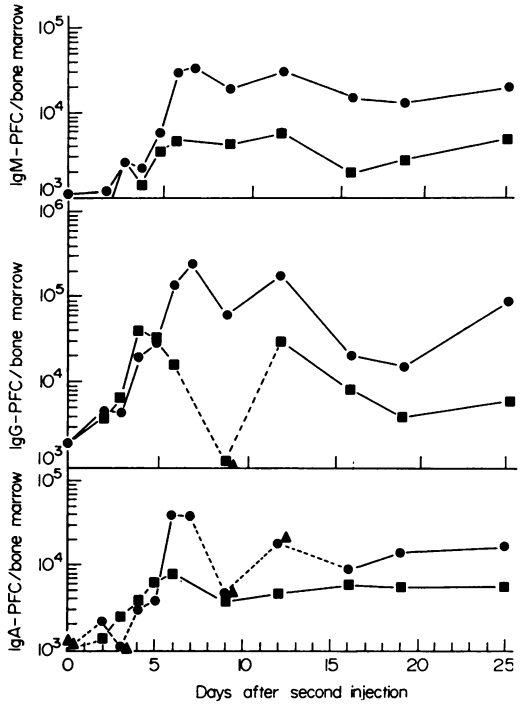


FIG. 3. Number of PFC in mouse bone marrow after two i.v. injections of $4 \times 10^8$ SRBC. The second injection was given 5 weeks after the first. (■) Sham-splenectomy and (●) splenectomy. Sham-splenectomy and splenectomy were performed 1 day before the second injection of SRBC. Where (▲) is added to an experimental point it means that the number of IgG-PFC or IgA-PFC above the level of IgM-PFC was not significant.

## SECONDARY RESPONSE OF BONE MARROW AFTER SPLENECTOMY

In the literature it has been suggested that PFC can migrate from spleen to bone marrow (Langevoort *et al.*, 1963; van Furth *et al.*, 1966; Chaperon, *et al.*, 1968; Hijmans and Schuit, 1972). To find out whether or not this migration causes the activity of the bone marrow during secondary response in our system, an experiment was performed in which mice were splenectomized 1 day before the second intravenous injection of SRBC.

Fig. 3 shows that splenectomy is not able to prevent the activity of the bone marrow in the secondary response. Once again, peripheral lymph nodes, mesenteric lymph node and Peyer's patches did not show an increase of the number of PFC. Therefore the PFC activity in bone marrow appears to be caused by a development of PFC *in situ*.

It is noteworthy that the bone marrow of the splenectomized animals as compared with the sham-splenectomized ones demonstrates an even higher PFC activity. Two ex-

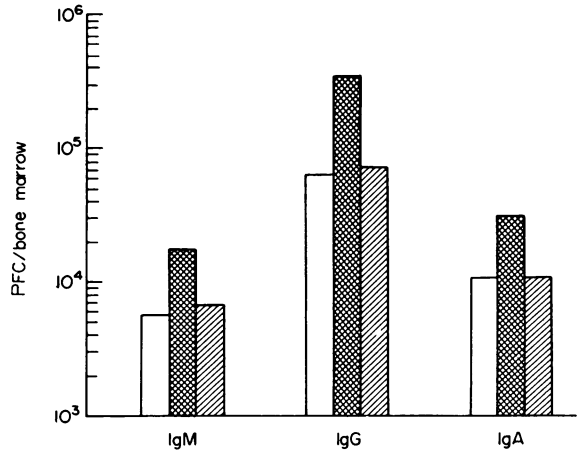


FIG. 4. Number of PFC in mouse bone marrow 6 days after the last of two i.v. injections of $4 \times 10^8$ SRBC. The second injection was given 5 weeks after the first. Open columns, sham-splenectomy; cross-hatched columns, splenectomy; hatched columns, splenectomy plus an i.v. injection of 0·5 ml hyperimmune anti-SRBC-serum. Operations were performed 1 day before the second injection of SRBC. The serum was administered 2 days after the second antigen injection.

planations for this phenomenon can be postulated. One explanation includes the absence of suppressing antibodies from the spleen. A regulatory effect of antibodies on the PFC response has been described (Uhr and Möller, 1968). This possibility was tested by giving sham-splenectomized mice an intravenous injection of 0·5 ml hyperimmune anti-SRBC-serum on the second day after the second antigen administration. The titre observed in normal mice injected with this amount of anti-SRBC-serum was $2^{14}$. Fig. 4 shows that the administration of hyperimmune anti-SRBC-serum suppressed the bone marrow PFC response. Another explanation could be that in the absence of the spleen more antigen is taken up by the bone marrow. The influence of the antigen supply on the bone marrow PFC activity was studied by challenging two groups of intact mice with doses of $4 \times 10^8$ and $10^9$ SRBC respectively. As shown in Fig. 5 an increased amount of SRBC

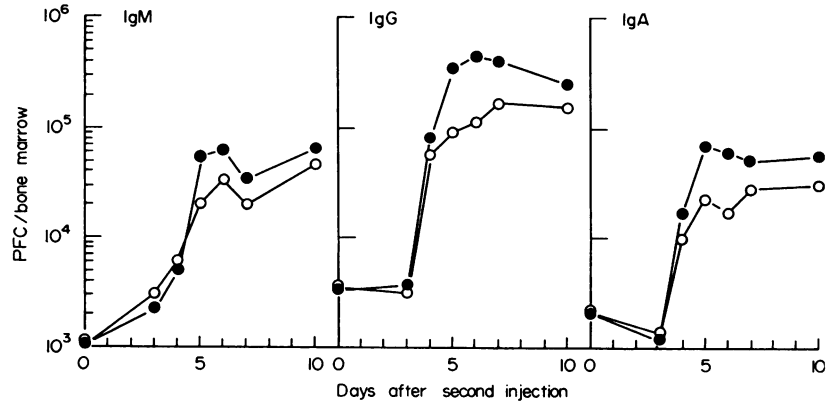


FIG. 5. Number of PFC in mouse bone marrow after two i.v. injections of SRBC. Both groups received a first injection of $4 \times 10^8$ SRBC. One group (●) received a second injection of $10^9$ SRBC; the other group (○) was inoculated with $4 \times 10^8$ SRBC.

stimulated the bone marrow to a somewhat greater activity. It is questionable whether this greater activity of the bone marrow is a direct consequence of this increased antigen supply. It is possible that the larger amount of SRBC reduces the concentration of free circulating anti-SRBC-antibodies from the primary response, thereby diminishing the negative feed back of these antibodies upon the secondary response of the marrow.

## DISCUSSION

The bone marrow is generally considered to be a primary lymphoid organ, since among its progeny are lymphocytes which are of major importance for the immunological capacity of other lymphoid organs (Miller, 1968). *In vitro* experiments (Thorbecke and Keuning, 1953, 1956; Askonas and Humphrey, 1958; Askonas and White, 1956; Langevoort *et al.*, 1963; Askonas *et al.*, 1965; Fleming *et al.*, 1967) demonstrate that bone marrow itself is capable of antibody synthesis. In these experiments the authors used bone marrow of primed organisms. Bone marrow of unprimed organisms is unable to respond to antigenic stimulation *in vitro* (Thorbecke and Keuning, 1956; Langevoort *et al.*, 1963; McMillan *et al.*, 1972).

Calculations of the contribution of the bone marrow to the overall production of immunoglobulins suggest that the marrow may be a major source of immunoglobulins (Askonas and White, 1956; Hijmans and Schuit, 1972; McMillan *et al.*, 1972).

Experiments in which the activity of the bone marrow was tested with the haemolytic plaque assay have been reported, with mice used as experimental animals (Friedman, 1964; Eidinger and Pross, 1967; Chaperon *et al.*, 1968; Mellbye, 1971; Cohen, 1972; Anderson and Dresser, 1972). Calculation of the total amount of PFC in mouse bone marrow requires an estimation of the number of bone marrow cells in the entire animal. For our calculations we made use of the results of Chervenick *et al.* (1968). These authors demonstrated with $^{59}$Fe-labelling that one femur of a mouse contains 5·9 per cent of its bone marrow. Although the distribution of $^{59}$Fe is not necessarily representative for the distribution of lymphocytes, it gives a clue for the total amount of bone marrow. Using this estimation we found a small number of PFC in the bone marrow during the late phase of the primary response after intravenous immunization (Fig. 1). This observation is consistent with the results of others (Eidinger and Pross, 1967; Chaperon *et al.*, 1968; Mellbye, 1971; Anderson and Dresser, 1972) using other routes of immunization. However, during the secondary response the bone marrow is particularly active, even in the early phase (Fig. 2). In a comparable experimental approach Eidinger and Pross (1967) found a small number of IgG-PFC in the bone marrow during the early response after a second intradermal immunization.

It is worth noting that the bone marrow needs a longer period of induction than the spleen. It was also demonstrated in rabbits that non-splenic sites have a longer lag time than the spleen after intravenous injections of SRBC (Taliaferro and Taliaferro, 1952). The spleen appears to synthesize antibody more rapidly after intravenous injections of SRBC than the bone marrow.

From the 9th to the 13th day the bone marrow contains approximately as many IgM-, IgG- and IgA-PFC as the spleen. From the 13th day on the major part of the total number of IgG-PFC is found in the bone marrow. This strong IgG-PFC activity corresponds well with the other results published (van Furth *et al.*, 1966; McMillan, 1972). It has been suggested in the literature (Langevoort *et al.*, 1963; van Furth *et al.*, 1966; Chaperon *et al.*, 1968;

Hijmans and Schuit, 1972) that antibody-forming cells from the peripheral lymphoid organs home to the bone marrow. Because only spleen and bone marrow showed a PFC activity during the secondary response in our experiments we could test this suggestion by splenectomy before the second injection of SRBC.

It was demonstrated that splenectomy does not prevent the secondary response of the bone marrow. Probably the bone marrow PFC originate *in situ*, because after splenectomy the mesenteric lymph node, the peripheral lymph nodes and Peyer's patches did not show a PFC-response.

The question then arises as to why the bone marrow is barely capable of antibody synthesis in the primary response, whereas a PFC activity in the secondary response can be clearly demonstrated. The most plausible explanation is that memory cells appear in the bone marrow after antigenic stimulation, so that a subsequent dose of the antigen triggers the development of B memory-cells into PFC. Experiments confirming this explanation will be described in a following paper. Finally it is interesting that the bone marrow compensates for the loss of the spleen by a greater number of PFC in comparison with the sham-splenectomized animals. The absence of regulating antibodies from the spleen (Fig. 4) is probably the cause of this greater activity of the bone marrow.

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